Comparative evaluation of two severe acute respiratory syndrome (SARS) vaccine candidates in mice challenged with SARS coronavirus

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Two different severe acute respiratory syndrome (SARS) vaccine strategies were evaluated for their ability to protect against live SARS coronavirus (CoV) challenge in a murine model of infection. A whole killed (inactivated by β-propiolactone) SARS-CoV vaccine and a combination of two adenovirus-based vectors, one expressing the nucleocapsid (N) and the other expressing the spike (S) protein (collectively designated Ad S/N), were evaluated for the induction of serum neutralizing antibodies and cellular immune responses and their ability to protect against pulmonary SARS-CoV replication. The whole killed virus (WKV) vaccine given subcutaneously to 129S6/SvEv mice was more effective than the Ad S/N vaccine administered either intranasally or intramuscularly in inhibiting SARS-CoV replication in the murine respiratory tract. This protective ability of the WKV vaccine correlated with the induction of high serum neutralizing-antibody titres, but not with cellular immune responses as measured by gamma interferon secretion by mouse splenocytes. Titres of serum neutralizing antibodies induced by the Ad S/N vaccine administered intranasally or intramuscularly were significantly lower than those induced by the WKV vaccine. However, Ad S/N administered intranasally, but not intramuscularly, significantly limited SARS-CoV replication in the lungs. Among the vaccine groups, SARS-CoV-specific IgA was found only in the sera of mice immunized intranasally with Ad S/N, suggesting that mucosal immunity may play a role in protection for the intranasal Ad S/N delivery system. Finally, the sera of vaccinated mice contained antibodies to S, further suggesting a role for this protein in conferring protective immunity against SARS-CoV infection.

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INTRODUCTION

Severe acute respiratory syndrome (SARS) emerged as a new disease threat in late 2002 and spread to several countries with considerable loss of life (8096 cases, 774 deaths; http://www.who.int/csr/sars/country/table2004_04/_21/en/index.html). The aetiological agent of SARS is a new human coronavirus (CoV) identified by its unique genome sequence (Marra *et al.*, 2003; Rota *et al.*, 2003) and by experimental infection of macaques to fulfil Koch's postulates (Fouchier *et al.*, 2003).

Currently, there is no effective treatment for SARS. Prevention through contact-reduction or transmission-blocking measures has been the only means available to modify the devastating impact of this illness. Prevention through vaccination would be an attractive alternative that is less reliant on individual case detection to be effective. No vaccines are currently licensed for any of the human CoVs, but effective vaccines have been produced for some animal CoVs, such as certain strains of *Infectious bronchitis virus* (poultry), *Bovine coronavirus* and *Canine coronavirus* (Cavanagh, 2003; Enjuanes *et al.*, 1995; Pratelli *et al.*, 2003; Saif, 2004; Takamura *et al.*, 2002). Individuals convalescing from SARS develop high titres of neutralizing antibodies (Tan *et al.*, 2004) and the appearance of antibodies coincides with the onset of resolution of SARS pneumonia (Liu *et al.*, 2004; Woo *et al.*, 2004). Thus, there is some optimism that an effective vaccine against SARS-CoV may also be possible.

Coronavirus spike (S) proteins have long been known to be a major determinant in coronavirus pathogenesis, given that this viral protein interacts with cellular receptors as well as contains determinants for eliciting a protective immune response (Enjuanes *et al.*, 1995; Navas-Martin & Weiss, 2003). Consequently, the SARS-CoV S glycoprotein, shown to be responsible for receptor binding to the cellular angiotensin-converting enzyme 2 (ACE2), is an attractive target for both vaccine and therapeutics development (Li *et al.*, 2003). This is strongly supported by the finding that a human mAb that binds to the N terminus of the S protein potently neutralizes SARS-CoV infection and inhibits syncytia formation through blocking of receptor binding (Berger *et al.*, 2004). Moreover, the S protein has been shown to induce serum neutralizing antibodies and to confer protective immunity against SARS-CoV challenge in mice and African green monkeys (Bisht *et al.*, 2004; Bukreyev *et al.*, 2004; Yang *et al.*, 2004). Studies from other animal CoV vaccines have also shown that the CoV nucleocapsid (N) protein, which encapsidates the viral genome, may represent another antigen candidate for vaccine development (Antón *et al.*, 1996; Olsen, 1993). Although antibodies to CoV N proteins have no virus-neutralizing activity *in vitro*, there is evidence that the protein may provide *in vivo* protection by induction of cell-mediated immunity (Enjuanes *et al.*, 1995; Stohlman *et al.*, 1995; Wesseling *et al.*, 1993). The N protein has been shown to generate CoV-specific $CD8^+$ T cells (Boots *et al.*, 1991; Seo *et al.*, 1997; Stohlman *et al.*, 1993, 1995) and to provide protection in animals following infection (Collisson *et al.*, 2000; Seo *et al.*, 1997).

Several potential strategies can be considered for vaccination against SARS-CoV, including a whole killed virus (WKV) vaccine, a live-attenuated SARS-CoV vaccine, a viral vector such as adenovirus or *Vaccinia virus* expressing SARS-CoV genes, recombinant SARS-CoV proteins and DNA-based vaccines (reviewed by See *et al.*, 2005). In this report, two SARS vaccine approaches were developed in parallel and evaluated for their efficacy in a murine SARS model by the SARS Accelerated Vaccine Initiative described elsewhere (Finlay *et al.*, 2004). We report the first direct comparison of a whole killed SARS-CoV vaccine and a combination of attenuated adenoviruses, one expressing SARS-CoV S protein and the other expressing the N protein (collectively called Ad S/N), for their ability to protect against live SARS-CoV challenge *in vivo*. Vaccine candidates were developed in parallel and evaluated for immunogenicity and efficacy against SARS-CoV infection in a murine model previously demonstrated to support virus replication (Hogan *et al.*, 2004).

METHODS

WKV vaccine. The Toronto-2 (Tor2) SARS-CoV strain (Marra *et al.*, 2003) was kindly provided by Dr Tim Booth, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada (Public Health Agency of Canada). SARS-CoV Tor2 strain was passaged in Vero E6 cells, purified by ultracentrifugation and subsequently inactivated with β-propiolactone (BPL) in a biosafety-level 3 facility, as described previously (Zakhartchouk *et al.*, 2005a). Inactivated virus was stored at $-80\,^{\circ}C$ until use. BPL-inactivated virus (approx. 1 mg ml^{-1} by Coomassie blue dye assay) was diluted in 0·1 M PBS, pH 7·0 (without divalent cations), to a protein concentration of 250 μg ml^{-1}. For animals receiving inactivated virus in alum adjuvant (2 % Alhydrogel; Accurate Chemical & Scientific Corp.), BPL-inactivated virus (500 μg ml^{-1}) was diluted in PBS and mixed with 2 % Alhydrogel diluted 1 : 4 for $\geqslant$6 h at 4 °C to give a final concentration of 250 μg virus and 2·5 mg Alhydrogel ml^{-1}.

Adenovirus-vectored SARS vaccine. The full-length SARS-CoV N gene (nt 28120–29482 of the SARS-CoV genome, GenBank accession no. NC_004718; Marra *et al.*, 2003) was amplified from an isolate of the Tor2 strain by RT-PCR and inserted into the pCR-Blunt II-TOPO vector (Invitrogen) to generate pTOPO-N. The N gene fragment was then isolated from pTOPO-N and inserted into the Ad5 shuttle plasmid pDC516(io) (AdMax Hi-IQ kit J; Microbix) downstream of a modified murine cytomegalovirus immediate-early promoter and upstream of the simian virus 40 polyadenylation signal. The S gene (corresponding to nt 21492–25259 of the SARS-CoV

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genome) was PCR-amplified from the plasmid pIR-GP SARS-V5, obtained from Nabil Seidah (Institut de Recherches Cliniques de Montreal, Quebec, Canada) and inserted into pDC516(io) to generate the shuttle plasmid pDC516(io)SARS-S. 293-IQ cells (Microbix) (Matthews *et al.*, 1999) were co-transfected with the genomic plasmid pBHGfrtdE1,3FLP (Ng *et al.*, 2000) and the shuttle plasmid pDC516(io)SARS-N or pDC516(io)SARS-S to generate AdSARS-N complete (shortened to Ad N) and AdSARS-S (shortened to Ad S), respectively, by Flp recombinase-mediated site-specific recombination. These vectors were amplified, purified and titrated by plaque assay as described by Hitt *et al.* (2005) with no evidence of heterologous insert instability. The Ad N vector described here carried the same N gene as Ad5-N-V reported recently (Zakhartchouk *et al.*, 2005b), but differed in the promoter (modified murine cytomegalovirus promoter in this Ad N construct) used in controlling N gene transcription. Expression of the SARS-CoV N or S protein was confirmed by Western blot analysis of lysates from human embryonic kidney (HEK293) or HeLa cells infected with the adenovirus-based vector for 24 h, using convalescent sera from SARS patients as a source of antibodies.

Molecular cloning, expression and purification of SARS-CoV N protein in *Escherichia coli*. DNA fragments containing the SARS-CoV N gene were generated by RT-PCR using SARS-CoV genomic RNA as the template. In order to subclone the PCR product as an *Nde*I–*Eag*I fragment into the pET30b(+) vector (Novagen), the following forward and reverse primers were used: 5′-GAATTCCATATGTCTGATAATGGACCCAATC-3′ and 5′-GAAA-GCCGGCCGATGCCTGAGTTGAATCAGCAG-3′. The PCR mixture contained 2 mM $MgSO_4$, 200 μM dNTPs, 2·5 U *Pfu* DNA polymerase and 25 pmol each oligonucleotide primer. PCR cycling conditions were as follows: one cycle of 95 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 2 min. Plasmids containing the N gene were then transformed into *E. coli* strain BL21 (DE3). SARS-CoV N protein expression was induced in transformed BL21 (DE3) host cells by adding 1 mM IPTG for 4 h. The expressed N protein, containing an N-terminal histidine tag, was subsequently purified by using the His Bind purification system (Novagen) and confirmed by SDS-PAGE.

S protein fragment (aa 318–510). A mammalian expression vector consisting of a codon-optimized gene encoding the SARS-CoV S protein aa 318–510 fragment (kindly provided by David Kelvin, University of Toronto, Canada) downstream of the mammalian transin-secretion signal peptide, a protein A purification tag (Sanchez-Lopez *et al.*, 1988), and a tobacco etch virus (TEV) protease-cleavage site was generated by using the pIRESpuro3 plasmid (Clontech). The secreted N-terminal fusion protein was purified from the culture medium of stably transformed HEK293T cells by IgG affinity chromatography. Following TEV cleavage, the S protein was further purified by hydrophobic-interaction chromatography, cation-exchange chromatography and gel filtration. Purity was assessed by Coomassie blue staining following SDS-PAGE.

Mice. Six-week-old female 129S6/SvEv mice, free of adventitious rodent pathogens, ectoparasites and endoparasites, were purchased from Taconic Farms (Germantown, NY, USA). Mice were housed in groups of eight in polysulfone microisolator cages on Sani-Chip bedding (PJ Murphy Forest Products). They were provided with food and water *ad libitum*. All procedures were in accordance with the NRC (1996), the Animal Welfare Act and the CDC NIH (1999).

Mice immunizations. Two groups of 129S6/SvEv mice were used in this study. Group 1 mice ($n=5$ per vaccine group) were immunized on day 0 and week 4 prior to sacrifice at week 7. These mice were examined for both humoral and cell-mediated immune responses as a result of immunization with the different SARS vaccines. Group 2 mice ($n=8$ per vaccine group) were immunized as above, but were subsequently challenged with live SARS-CoV Tor2 at week 7 (see below). For immunizations on day 0, mice were vaccinated either with a combination of adenoviruses, one expressing SARS-CoV S protein and the other expressing SARS-CoV N protein (collectively called Ad S/N), or with the WKV vaccine in the presence or absence of alum. Specifically, 129S6/SvEv mice were immunized in one the following manners: (i) subcutaneously with 0·2 ml 0·1 M PBS, pH 7·0 (without divalent ions); (ii) subcutaneously with WKV alone, consisting of 50 μg inactivated virus in 0·2 ml 0·1 M PBS; (iii) subcutaneously with WKV vaccine plus alum, consisting of 50 μg inactivated virus plus 500 μg Alhydrogel in 0·2 ml 0·1 M PBS; (iv) intranasally with adenovirus-based SARS-CoV S and N vectors (3×10^8 p.f.u. each) in a total volume of 30 μl (Ad S/N IN); (v) intramuscularly with adenovirus-based SARS-CoV S and N vectors (3×10^8 p.f.u. each) into the hind leg (Ad S/N IM); (vi) intranasally or intramuscularly with a control adenovirus 5 (6×10^8 p.f.u.) that lacks SARS-CoV genes (Ad-Ctrl). Before intranasal vaccination with the recombinant adenovirus SARS vaccines, each mouse was anaesthetized with isoflurane. Four weeks after the initial immunization, mice were reimmunized with the same vaccine at the same dose and monitored until week 7.

SARS-CoV challenge studies. Following vaccination at 0 and 4 weeks, group 2 mice were challenged at 7 weeks with the SARS-CoV Tor2 strain. For challenge experiments, eight mice from each vaccine group were anaesthetized with isoflurane and infected via intranasal inhalation with 1×10^6 p.f.u. SARS-CoV Tor2 in a total volume of 30 μl. On day 3 post-challenge, half ($n=4$) of the animals were euthanized with CO_2 and necropsied. The remaining four animals were sacrificed in the same way on day 7 post-challenge.

Collection of blood and lung tissues. Blood was collected from each mouse via retro-orbital puncture during the following time periods: prior to the first immunization, week 4 (pre-boost) and week 7 (pre-challenge). Blood samples were processed by clotting for 30 min at room temperature and centrifuged to remove cellular debris, and the resulting sera were stored at −80 °C. The lungs from each mouse were removed aseptically and homogenized in PBS as described below. Fifty microlitres of the lung homogenate was mixed with 450 μl TRIzol reagent and stored at −80 °C for RNA analysis. The remaining lung homogenate was stored at −80 °C for virus titration.

Virus titration. Frozen lung (previously unwashed) samples were homogenized in PBS containing penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin (50 μg ml^{-1}). Lung homogenates were then diluted serially (half-log) in Dulbecco's minimal essential medium containing 2 % heat-inactivated fetal bovine serum (Atlanta Biologicals) and antibiotics (penicillin and streptomycin) before addition to 90–95 % confluent Vero E6 monolayers in 96-well plates. Cells were monitored for cytopathic effect (CPE) in positive-control wells. After incubation for 48 h at 37 °C, 5 % CO_2, CPE was measured by the addition of neutral red to the wells and measurement of A_{540}. $TCID_{50}$ was determined by a 50 % reduction in CPE as described previously (Guo *et al.*, 2004; Schmidt, 1989; Smee *et al.*, 2001). Viral titres were expressed as $\log_{10}$ $TCID_{50}$ ml^{-1} for lung homogenates.

Virus neutralization assay. Twofold dilutions of heat-inactivated serum were tested for the presence of antibodies that would neutralize the infectivity of 100 $TCID_{50}$ of SARS-CoV in Vero E6 cell monolayers as described previously (Zakhartchouk *et al.*, 2005a). The CPE of SARS-CoV on Vero E6 cell monolayers was read on day 3. The dilution of serum that completely inhibited CPE in 50 % of the wells was calculated as described previously (Reed & Muench, 1938).

Gamma interferon (IFN-γ) ELISPOT assay. MultiScreen-IP (Millipore) 96-well plates were coated overnight with rat anti-mouse

IFN-γ (Pharmingen) at 4 °C. Plates were washed with sterile PBS and incubated in triplicate with 2×10^5 murine splenocytes per well in RPMI 1640. Recombinant N protein was isolated as described above and added to a final concentration of 10 μg ml^{-1}. After incubation for 40 h at 37 °C, plates were washed six times with PBS containing 0·05 % Tween 20 and incubated overnight with 100 μl biotinylated rat anti-mouse IFN-γ (Pharmingen) per well. After washing, streptavidin–alkaline phosphatase was added and the plates were developed and read as described previously (Zakhartchouk *et al.*, 2005a).

Western blot analysis using pooled mice sera. Purified bacterial N protein (0·2 μg) and either 0·5 or 5 μg truncated mammalian S protein (aa 318–510) were prepared in SDS sample buffer, separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Specific proteins on the membranes were visualized as described previously (Zakhartchouk *et al.*, 2005a).

SARS-CoV-specific ELISA. Total SARS-CoV-specific IgG, IgG isotypes and IgA titres in sera from immunized mice were measured by ELISA. Each well of a 96-well plate was coated overnight with 0·1 ml purified inactivated SARS-CoV at a concentration of 1 μg ml^{-1}. Washing of plates, addition of sera and colour development were performed as described previously (Zakhartchouk *et al.*, 2005a).

RT-PCR assay for SARS-CoV RNA. Fifty microlitres of lung homogenate was placed in TRIzol reagent and processed as described previously (Hogan *et al.*, 2004). RNA was isolated with an RNeasy kit (Qiagen) and tissue RNA was quantified with RiboGreen (Molecular Probes). DNase treatment was performed to eliminate the remaining DNA in the RNA samples. The SARS-CoV genome was detected with LUX primer sets for the SARS-CoV N domain and the Superscript III Platinum One-step Quantitative RT-PCR System (Invitrogen) in a LightCycler (Roche) as described previously (Hogan *et al.*, 2004). To confirm the RT-PCR results, RT-PCR products from some of the study samples were verified by gel electrophoresis. The results ranged from less than one copy to up to 10 000 copies per reaction.

Statistical analyses. Statistical significance was assessed by using the non-parametric Wilcoxon rank sum test (Hollander & Wolfe, 1973). Differences between mean values for the vaccine groups were considered significant if the P value (two-tailed) was $\leqslant 0{\cdot}05$.

RESULTS

Neutralizing-antibody responses to SARS vaccines

In contrast to the PBS control, group 1 mice vaccinated with the WKV vaccine demonstrated detectable serum neutralizing-antibody titres at 4 weeks, and by 7 weeks the titres had increased 40-fold (Fig. 1a). The addition of alum to the WKV vaccine preparation did not increase serum neutralizing-antibody levels significantly at either time period (Fig. 1a). Mice given Ad S/N IM showed similar serum neutralizing-antibody titres at weeks 4 and 7, but overall titres were 25-fold lower than those observed in the sera of mice given WKV with or without alum (Fig. 1a). In contrast, the neutralizing-antibody titres in the sera of Ad S/N IN-vaccinated mice were low during the first 4 weeks, but increased 11-fold at the end of 7 weeks, albeit with overall titres that were lower than those found in Ad S/N IM sera (Fig. 1a). No serum neutralizing-antibody responses could be detected within the 7 week period in mice immunized with the control adenovirus (Ad-Ctrl) (Fig. 1a).

Total SARS-CoV-specific IgG titres in sera from weeks 4 and 7 were determined by ELISA using inactivated SARS-CoV as the capture antigen. As shown in Fig. 1(b), no significant difference in total SARS-CoV-specific IgG levels was observed between WKV alone and WKV plus alum at either week 4 or week 7, supporting the data in Fig. 1(a). However, a 19-fold difference ($P=0{\cdot}02$) in total SARS-CoV-specific IgG levels was observed at weeks 4 and 7 in the sera of mice vaccinated with WKV alone or with WKV plus alum (Fig. 1b). The total SARS-CoV-specific IgG levels measured from week 7 sera of either Ad S/N IM- or Ad S/N IN-vaccinated mice did not differ statistically from those of week 4 sera and were approximately sevenfold lower than those induced by the WKV plus alum vaccine at week 7 (Fig. 1b). No SARS-CoV-specific IgG was detected in the sera of mice vaccinated with either PBS or Ad-Ctrl at either time period. The SARS-CoV-specific IgG subclasses were also determined in week 7 sera. Fig. 1(c) shows that SARS-CoV-specific IgG2a levels in week 7 sera were significantly higher than IgG1 levels in response to WKV alone ($P=0{\cdot}008$), WKV plus alum ($P=0{\cdot}03$) or Ad S/N given either intramuscularly ($P=0{\cdot}002$) or intranasally ($P=0{\cdot}0001$). The addition of alum to WKV significantly augmented ($P=0{\cdot}008$) the SARS-CoV-specific IgG1, but not the IgG2a, response (Fig. 1c). Our results showed that the WKV alone and Ad S/N IM vaccines induced similar levels of SARS-CoV-specific IgG isotypes at week 7. Additionally, we analysed the sera of all vaccinated mice by ELISA for the presence of SARS-CoV-specific IgA, which would indicate vaccine stimulation of mucosal immunity. The results in Fig. 1(d) indicated that sera from mice vaccinated with Ad S/N IN showed a greater than 3 log increase in SARS-CoV-specific IgA titres compared with Ad-Ctrl-vaccinated mice at weeks 4 and 7. No detectable SARS-CoV-specific IgA was found in the sera of mice immunized with either WKV (with or without alum) or Ad S/N IM for up to 7 weeks (Fig. 1d).

To determine the SARS-CoV proteins recognized by the sera of mice vaccinated with either WKV or Ad S/N at week 7, Western blot analysis was performed on purified recombinant S or N proteins. In contrast to mice receiving either PBS or Ad-Ctrl, bands corresponding to the molecular masses of the S fragment (Fig. 2a) and the N protein (Fig. 2b) were detected by pooled sera from mice vaccinated with WKV, WKV plus alum, Ad S/N IM or Ad S/N IN. Interestingly, a consistently stronger immunoreactivity towards both the S and N proteins was evident in the sera of mice vaccinated with WKV plus alum compared with WKV alone (Fig. 2a and b, lanes 2 and 3), suggesting that alum may either enhance the response of this class of antibody or increase its avidity. In addition, the immunoreactivity of sera from Ad S/N IM- or Ad S/N IN-vaccinated mice to recombinant S protein was much weaker than that observed with WKV sera and required 10-fold more antigen to visualize the immunoreactive S protein band (Fig. 2a).

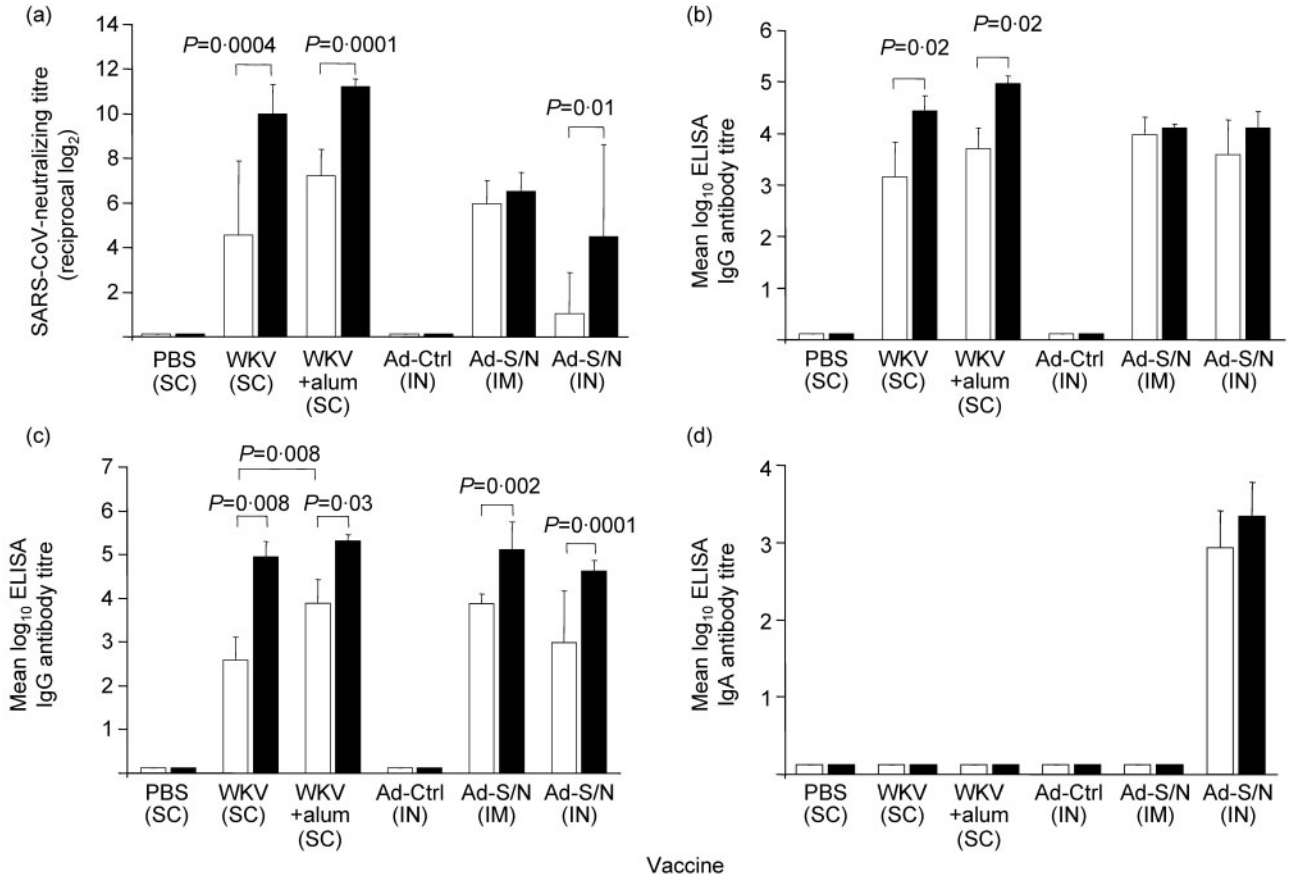

Fig. 1. Immunogenicity responses of group 1 mice to SARS vaccines. (a) Neutralizing-antibody titres (mean ± SD for five mice) of week 4 (empty bars) and 7 (filled bars) sera from mice vaccinated as indicated. (b) Total SARS-CoV-specific IgG titres (mean ± SD for five mice) are shown for week 4 (empty bars) and 7 (filled bars) sera of controls and vaccinated mice. (c) Titres of SARS-CoV-specific IgG1 (empty bars) and IgG2a (filled bars) subclasses (mean ± SD for five mice) in the sera of control and vaccinated mice 7 weeks after first immunization. (d) SARS-CoV-specific IgA titres in week 4 (empty bars) and 7 (filled bars) sera of mice vaccinated as indicated.

Collectively, our results indicate that the S and N proteins in the WKV and Ad S/N preparations were able to induce a readily quantifiable humoral response.

Cell-mediated immune responses to WKV and Ad S/N

The types of immune response elicited by WKV or Ad S/N after initial immunization and boost were evaluated further by measuring the secretion of IFN-γ by mouse splenocytes stimulated *in vitro* with recombinant SARS-CoV N protein. Fig. 3 shows that, when stimulated with N protein, splenocytes from mice vaccinated with either WKV or Ad S/N secreted significantly higher levels ($P=0{\cdot}002$) of IFN-γ compared with splenocytes from control mice. As indicated in Fig. 3, vaccination of mice with Ad S/N IM resulted in the highest number of IFN-γ-secreting splenocytes, followed by WKV alone, Ad S/N IN and WKV plus alum, in descending order.

Reduction of lung viral load in mice immunized with the Ad S/N or WKV vaccine

We assessed the efficacy of two SARS vaccines by using the 129S6/SvEv mouse model as described previously (Hogan *et al.*, 2004). From the vaccination period to the time of SARS-CoV challenge, mice appeared normal in activity and appetite and showed no signs of adverse reaction to any of the vaccines at the administration site. Viral titres were assessed in lung homogenates during the early and late courses of infection by using a CPE assay. Pilot studies with PBS showed that SARS-CoV titres in the murine respiratory tract were highest at day 3 post-challenge and dropped dramatically to undetectable levels by day 7 post-challenge (Hogan *et al.*, 2004). SARS-CoV replication in the lungs was assessed in the group 2 mice by examining viral titres and RNA levels in lung homogenates. Fig. 4(a) shows that lung viral titres in mice immunized with WKV in the absence or presence of alum were reduced by 4 logs to undetectable levels compared with titres observed in the PBS control on

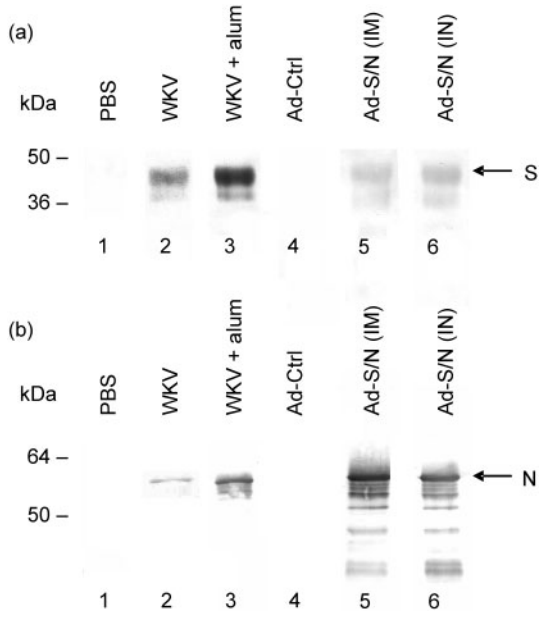

Fig. 2. Western blot analysis of week 7 sera from group 1 mice vaccinated as indicated and probed against the following antigens: (a) purified recombinant SARS-CoV S protein fragment (aa 318–510) (lanes 1–3, 0·5 μg protein; lanes 4–6, 5 μg protein); (b) recombinant His-tagged N protein (0·2 μg in each lane). Arrows indicate the position of the S and N proteins.

day 3 post-challenge ($P=0·02$). Lung viral titres in animals vaccinated with Ad S/N IN were also reduced significantly ($P=0·03$) compared with Ad-Ctrl-vaccinated animals harvested on day 3, albeit not as strongly as observed with WKV (Fig. 4a). A similar reduction trend, although not statistically significant, was observed when the lung viral titres of Ad S/N IM-vaccinated mice were compared with those of mice vaccinated with Ad-Ctrl (Fig. 4a). Viral titres in lung homogenates were below the assay detection limit for all vaccine groups on day 7 post-challenge. Levels of SARS-CoV RNA correlated well with the lung viral-titre data on day 3 post-challenge. Compared with the PBS control on day 3 post-challenge, levels of viral RNA were undetectable in lung homogenates of mice immunized with WKV alone or with WKV plus alum (Fig. 4b). A significant reduction ($P=0·02$) in the level of SARS-CoV RNA was also observed in day 3 post-challenge lung homogenates of mice given Ad S/N IN compared with animals vaccinated with Ad-Ctrl. SARS-CoV RNA was still detectable in lung homogenates at day 7 post-challenge for mice vaccinated with either PBS or Ad-Ctrl (Fig. 4b). By day 7 post-challenge, SARS-CoV RNA was undetectable for both groups of WKV-vaccinated animals (Fig. 4b). Residual low levels of SARS-CoV RNA were observed at day 7 post-challenge for mice immunized with Ad S/N IN or Ad S/N IM, but the levels were not significantly different from those of Ad-Ctrl-vaccinated mice (Fig. 4b).

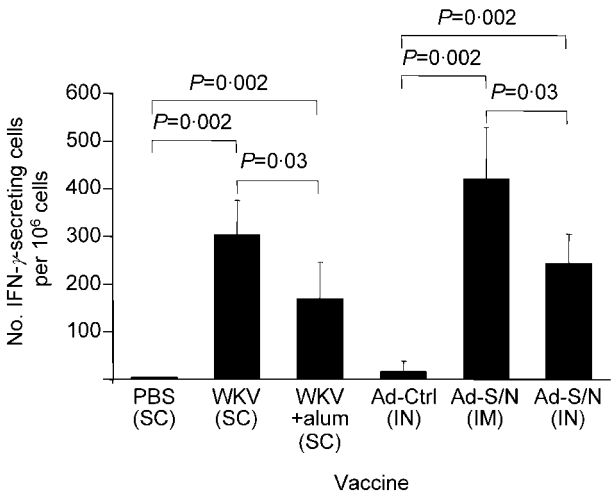

Fig. 3. Cellular immune responses of group 1 mice to SARS vaccines. The number of IFN-γ-secreting splenocytes per 10^6 cells from mice harvested 7 weeks after vaccination and stimulated *in vitro* with recombinant N protein for 24 h is shown. Results represent the mean ± SD (for triplicate wells) for groups of five mice.

DISCUSSION

We have described the first direct comparison of two SARS vaccines, WKV and Ad S/N, an adenovirus-based vaccine consisting of SARS-CoV S and N proteins. Induction of serum neutralizing antibodies and cellular immune responses was evaluated in order to define correlates of immunity for protection against SARS-CoV replication in lung tissue. Our findings showed that vaccination with WKV in mice induced much higher levels of serum neutralizing antibodies than Ad S/N given intramuscularly or intranasally. The addition of alum to the WKV vaccine preparation did not increase the serum virus-neutralizing activity significantly. In contrast, Ad S/N IM induced the highest cellular immune response among the tested vaccines, as measured by IFN-γ secretion by splenocytes of vaccinated mice following *in vitro* stimulation with recombinant N protein. The number of IFN-γ-secreting splenocytes in Ad S/N IM-immunized mice was significantly higher (1·5–2·5-fold) than those of mice vaccinated with Ad S/N IN, WKV alone or WKV plus alum. However, when evaluating protection against pulmonary virus replication in 129S6/SvEv mice, the WKV vaccine was more effective than Ad S/N administered intramuscularly or intranasally in reducing pulmonary SARS-CoV replication following challenge. No significant protection was observed in mice vaccinated with Ad S/N IM compared with the control animals. Our results suggest that humoral immunity, but not cellular immune responses, correlates well with the ability of WKV to protect against pulmonary SARS-CoV replication.

Recipients of the Ad S/N IN vaccine also demonstrated significantly reduced levels of SARS-CoV replication in the

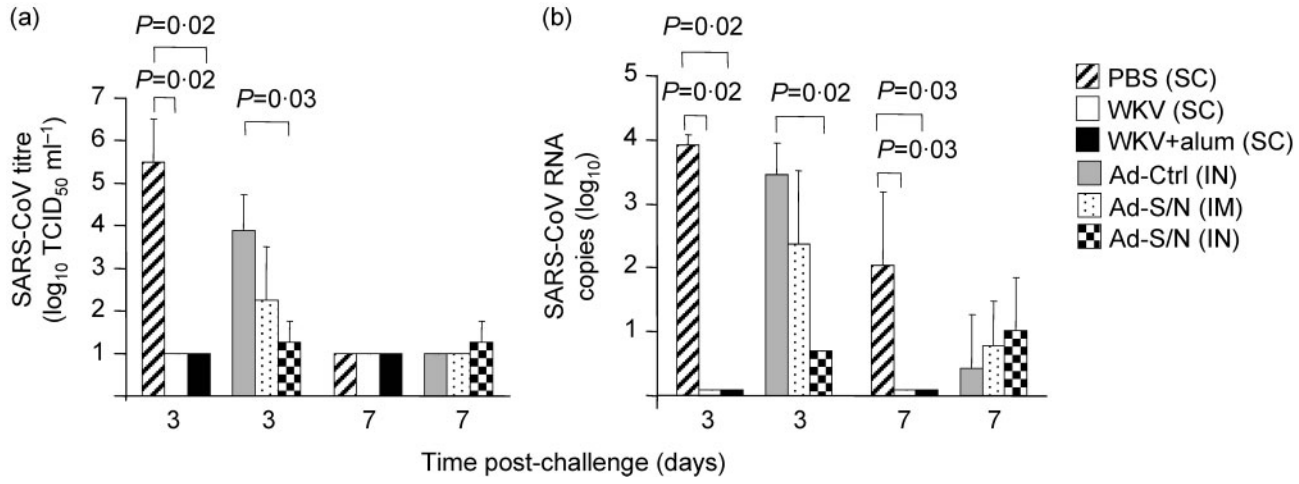

Fig. 4. Protection of group 2 mice from pulmonary SARS-CoV replication after immunization with SARS vaccines. (a) Viral titres (mean ± SD for four mice) were determined in homogenates of lungs harvested from vaccinated mice after challenge with SARS-CoV for the indicated number of days. The lower limit of virus detection was 1·0 $\log_{10}$ $TCID_{50}$ ml^{-1}. (b) SARS-CoV genome copy number (mean ± SD for four mice) was determined in homogenates of lungs from vaccinated mice challenged for the indicated number of days.

lungs, although protection was not as effective as the WKV vaccine. In contrast, the intramuscular route of the Ad S/N vaccine had only a limited effect in reducing pulmonary SARS-CoV replication, despite demonstrating high serum neutralizing-antibody titres prior to SARS-CoV challenge and the ability to induce a robust cellular immune response. In our study, the two intramuscular injections of the Ad S/N vaccine produced a strong SARS-CoV-specific IgG1 and IgG2a antibody response in mice, with IgG2a titres significantly higher than IgG1. The serum neutralizing-antibody levels in Ad S/N IN-vaccinated mice were lower than in animals immunized with Ad S/N IM. This result suggests that other factors, such as mucosal immunity (e.g. secretory IgA and/or mucosal T cells), may play an important role in limiting lung virus replication by the Ad S/N IN vaccine. The detection of serum SARS-CoV-specific IgA in mice vaccinated with Ad S/N IN, but not with Ad S/N IM or Ad-Ctrl, suggests that mucosal SARS-CoV-specific IgA does play an important role in conferring protection against SARS-CoV pulmonary replication. Moreover, the IFN-γ-secreting response observed in splenocytes of Ad S/N IN-vaccinated mice was more than twofold lower than that found in mice immunized with Ad S/N IM, indicating that cellular immune responses do not contribute to protection against SARS-CoV challenge as strongly as an antibody response, consistent with other studies (Yang *et al.*, 2004). Our result points to a potential approach whereby SARS-CoV infection could potentially be blocked at the primary site of entry (e.g. respiratory tract) by using an intranasal adenovirus-based vaccine (Bukreyev *et al.*, 2004; Peiris *et al.*, 2003).

There are several possible explanations as to why the WKV vaccine conferred more protection against pulmonary SARS-CoV replication than the Ad S/N vaccine. Firstly, the WKV vaccine induced higher serum virus-neutralizing activity than mice immunized with either Ad S/N IN or Ad S/N IM. Secondly, the route of administration (subcutaneous for WKV vaccine, intranasal or intramuscular for Ad S/N) or the vaccine dose may contribute to the protective differences observed between the WKV and Ad S/N vaccines. Thirdly, expression of the S protein from the Ad S/N construct may be lower than that of WKV. This was evident in Fig. 2(a), where sera from WKV-vaccinated mice showed a much stronger immunoreactivity towards the S protein than sera from Ad S/N-immunized animals. Indeed, we have recently constructed a single adenovirus vector containing codon-optimized S and N genes and found that the serum neutralizing-antibody titres induced by this construct, when given intranasally/intramuscularly to mice, were comparable to levels found in animals vaccinated with WKV (data not shown). Lastly, unlike the Ad S/N vaccine, which contains only genes that encode the N and S proteins, the WKV vaccine contains the complete complement of viral proteins present in the natural virion conformation and thus may induce a broader immune response. In support of this, others have reported that sera from mice vaccinated with inactivated SARS-CoV contain antibodies to a number of proteins including S, N, M and 3CL of the SARS-CoV Tor2 strain (Xiong *et al.*, 2004).

Several groups have developed SARS vaccines based on the SARS-CoV S protein as a target. A DNA-based vaccine (Buchholz *et al.*, 2004), a modified Ankara vaccinia virus (Bisht *et al.*, 2004) and a recombinant attenuated parainfluenza virus (Bukreyev *et al.*, 2004) containing the SARS-CoV S gene have been shown to induce serum neutralizing antibodies and to inhibit pulmonary virus replication in animals. These animal models for SARS include macaques

(Fouchier *et al.*, 2003; Kuiken *et al.*, 2003), African green monkeys (Bukreyev *et al.*, 2004; McAuliffe *et al.*, 2004), ferrets (Martina *et al.*, 2003), mice (Glass *et al.*, 2004; Subbarao *et al.*, 2004) and hamsters (Roberts *et al.*, 2005). Although all of the above animal models support virus replication, no single animal species has been shown to reproduce all of the clinical signs and lethality observed in humans infected with SARS-CoV. The importance of a humoral response to the S protein has been demonstrated by the protection of naïve mice from live SARS-CoV challenge after passive IgG transfer from immunized animals (Yang *et al.*, 2004). In contrast, antiserum to N protein has not been shown to contain neutralizing antibodies (Pang *et al.*, 2004). However, the N protein has been shown to be a vaccine candidate by inducing SARS-CoV antigen-specific T-cell and virus-neutralizing responses (Kim *et al.*, 2004; Zhu *et al.*, 2004). Several laboratories (He *et al.*, 2004; Qu *et al.*, 2005; Takasuka *et al.*, 2004; Tang *et al.*, 2004) have developed an inactivated whole SARS-CoV vaccine that can induce serum neutralizing antibodies when injected into mice, but whether these vaccines can confer protective immunity against live SARS-CoV challenge was not reported. Recently, a WKV vaccine has been shown to protect against pulmonary SARS-CoV replication in BALB/c mice, although characterization of the immune response was not reported (Stadler *et al.*, 2005). In this study, we also demonstrated that a whole killed SARS-CoV vaccine can induce serum neutralizing antibodies and can protect against SARS-CoV challenge in mice. We showed that protection from SARS-CoV infection by the WKV vaccine is associated with the induction of both an IgG1 and IgG2a immune response. The addition of alum, which selectively stimulates IgG1 immune responses (Kenney & Edelman, 2003), only increased serum SARS-CoV-specific IgG1 but not IgG2a responses, which is consistent with the findings of others (Takasuka *et al.*, 2004). In our study, total serum SARS-CoV-specific IgG and neutralizing-antibody levels were not enhanced by the addition of alum to the WKV vaccine preparation and protection from virus replication was not improved discernibly. In contrast, the immunoreactivity of pooled mouse sera towards both the full-length N protein and the S protein fragment was enhanced when the WKV vaccine formulation contained alum. These results suggest that the inclusion of alum in the WKV vaccine may lead to enhanced production of antibody to denatured protein. Interestingly, the S protein domain (aa 318–510) recognized by the sera of WKV-vaccinated mice in our studies is known to bind to the SARS-CoV functional receptor, ACE2 (Babcock *et al.*, 2004; Wong *et al.*, 2004; Xiao *et al.*, 2003). Antibodies present against this S domain may explain why sera from our WKV-vaccinated mice were able to block SARS-CoV-mediated CPE in Vero E6 cells.

In summary, this is the first description of two SARS vaccines evaluated head-to-head for their ability to induce immunogenicity and to reduce viral load in the murine respiratory tract. Our results showed that the WKV vaccine was more effective than the Ad S/N vaccine in the reduction of viral load in the respiratory tract of vaccinated mice after live SARS-CoV challenge, with the intranasal route of Ad S/N also providing significant protection. Such a direct comparison of SARS vaccines in an animal model will not only determine which vaccine strategy is more effective, but will also shorten the time lines for moving the best candidate forward into human testing.

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